Substitutions for Glu-537 of β -galactosidase from *Escherichia coli* cause large decreases in catalytic activity

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Glu-537 of β -galactosidase (EC 3.2.1.23) was replaced by Asp, Gln and Val using synthetic oligonucleotides. The k_{cat} values of the purified enzyme mixtures were reduced by about 100-fold for the Asp mutant, 30000–60000-fold for the Val mutant and 160000–300000-fold for the Gln mutant. The greatest differences in properties from the wild-type enzyme were found for the Asp-substituted enzyme: the K_m values increased (from 0.12 to 0.42 mM for *o*-nitrophenyl β -D-galactopyranoside and from 0.04 to 0.37 mM for *p*-nitrophenyl β -D-galactopyranoside), the K_i value for isopropyl β -D-galactopyranoside increased (from 0.11 to 0.30 mM), the stability to heat decreased and methanol did not act as an acceptor. The enzymes with the other two substitutions had properties similar to those of the wild-type. For

all three substituted enzymes, the inhibitory effects of the transition-state analogues (2-deoxy-2-amino-D-galactose and L-ribose) and the Mg^{2+} effects were similar to those of the normal enzyme. As all of the properties (except the k_{cat} values) of the Gln- and Val-substituted enzyme preparations were similar to those of the wild-type enzyme, the activities in those preparations were probably due to the presence of a few wild-type enzyme molecules (formed from misreads) among the substituted enzymes. The enzymes with Gln and Val substitutions appear to be totally inactive. The results obtained support a recent suggestion that Glu-537 is an important catalytic residue of β -galactosidase.

INTRODUCTION

β -Galactosidase (EC 3.2.1.23), the product of the *lacZ* gene of *Escherichia coli*, has 1023 amino acid residues, and is a good model for the study of enzymes that act on disaccharides. It has been suggested that Tyr-503 acts as an acid and base modulator in the catalytic mechanism (Ring et al., 1985, 1988; Ring and Huber, 1990), and that Glu-461 is involved in stabilizing the galactosyl intermediate (Herrchen and Legler, 1984), either as a galactosyl cation or by a covalent bond (or both). Gebler et al. (1992) have found that Glu-537 may also be essential for activity, and have proposed that it acts as a nucleophile during the catalytic reaction. They based this conclusion on their finding that β -D-2-deoxy-2-fluorogalactopyranose became covalently attached to Glu-537 when 2,4-dinitrophenyl β -D-2-deoxy-2-fluorogalactopyranoside was used as a substrate, and that this caused the enzyme to be inactive. With the advent of site-specific mutagenesis as a technique with a powerful ability to determine the effect of differences in structure on activity, it is essential that the importance of a residue that reacts in this way be confirmed by specific replacement. It is possible that the covalent attachment to Glu-537 and the subsequent inactivation was caused by the unphysiological substrate (2,4-dinitrophenyl β -D-2-deoxy-2-fluorogalactopyranoside) binding in an unusual fashion and that the reaction with Glu-537 does not normally occur. To confirm that Glu-537 is important, we replaced it with Asp, Gln or Val using synthetic oligonucleotide probes to single-stranded DNA that coded for β -galactosidase. The properties of the resulting enzyme preparations show that Glu-537 is essential for the activity of β -galactosidase from *E. coli*.

MATERIALS AND METHODS

Enzymes and chemicals

Restriction endonucleases (*Cla*I, *Sac*I, *Pst*I), and T4 ligase, etc., were from either Boehringer-Mannheim or Pharmacia LKB Biotechnology. A Sequenase kit, obtained from the U.S. Biochemical Corp., was employed according to the instructions supplied. A GeneClean kit, obtained from Bio 101, was used to remove the DNA cleanly from the agarose gels (Vogelstein and Gillespie, 1979). [α - 35 S]thio]dATP was from Du Pont-NEN. The oligonucleotide primers were synthesized at this university. Other chemicals were from Sigma, BRL or similar sources, and the purest grades available were always used.

Bacterial strains

The bacterial strains used were *E. coli* RZ1032 {HfrKL16 PO/45 *zbd-279::Tn10* [*lysA*(61–62)] *thi-1 relA1 spoT1 supE44 dut-1 ung-1*}, *E. coli* TG2 [*supE hsdΔ5 thi Δ(lac-proAB) Δ(SrL-recA)306::Tn10 (tet^r) F' (traD36 proAB⁺ lacI^q lacZΔ M15)] and *E. coli* Y1091 (protease negative) (Δ *lacU169 proA Δlon araD139 rpsL supF trpC22::Tn10 hsdR⁻ hsdM⁺*). Strains were stored as glycerol (25%) stocks (at -70°C), and melted on ice before use. Competent cells were prepared by the CaCl_2 method (Cohen et al., 1972). Other strains used were supplied with the commercial kits, and were used according to the instructions. Cells were cultured (37°C , 220 rev./min) in liquid Luria–Bertani media with 100 $\mu\text{g/ml}$ ampicillin. The mutants were screened with ampicillin (100 $\mu\text{g/ml}$), isopropyl β -D-galactopyranoside (IPTG)*

Abbreviations used: IPTG, isopropyl thio- β -D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; ONPG, *o*-nitrophenyl β -galactopyranoside; PNPG, *p*-nitrophenyl β -D-galactopyranoside.

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(2 mM) and 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) (50 μ g/ml).

Plasmids

Bluescript (2.96 kb) was from Stratagene. Plasmid pIP101 was a gift from Dr. Müller-Hill (University of Cologne, Cologne, Germany). Plasmid DNA was isolated using standard procedures (Sambrook et al., 1989). GeneClean was used to isolate DNA fragments (digested by restriction endonucleases) from agarose electrophoretic gel.

Construction and transformation of mutant genes

Plasmid pIP101 (containing the entire *lacZ* gene) was digested using the restriction endonucleases *Cl*aI and *S*acl to obtain a 1.1 kb fragment that contained the code for Glu-537. Bluescript was digested with the same restriction enzymes. The 1.1 kb fragment was isolated from 1% agarose gel using GeneClean, and ligated into Bluescript with T4 ligase. The uracil single-stranded DNA template was produced using *E. coli* RZ1032 (Kunkel et al., 1987). Oligonucleotide primers (23 bases) with the Glu-537 codon (GAA) replaced by GAC, CAA or GTA were used. The oligonucleotides were annealed to the uracil single-stranded DNA. Double-stranded plasmid DNA was generated with Klenow fragment, and replicated in *E. coli* TG2. The cells were screened in the presence of ampicillin, X-gal and IPTG. Single-stranded Bluescript DNA samples were sequenced to detect those that had the desired changes. The 1.1 kb fragments with the mutations were digested from the Bluescript, and ligated back into pIP101. The new pIP101 plasmids (containing the substitutions of the Glu-537 codon) were transformed into *E. coli* Y1091. After single colonies had been picked and cultured in Luria-Bertani medium containing ampicillin, the substituted β -galactosidases were purified.

Purification of mutant β -galactosidases

The purification procedures have been described previously (Cupples et al., 1990). The purity of the enzyme was examined by Phast-Gel SDS/PAGE. The buffer system used for the SDS/PAGE was 0.20 M Tricine, 0.20 M Tris and 0.55% SDS, pH 8.1. The native PAGE buffer system was 0.88 M L-alanine and 0.25 M Tris, pH 8.8. Standard Phastgel gels (0.45 mm thick) with continuous 10–15% gradients and with 2% cross-linking were used in each case. Protein concentration was determined by using the established 280 nm absorption coefficient of $2.09 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Assays

The assay mixture (25 °C) was composed of 1.0 ml of Tes buffer (30 mM, pH 7.0) containing 1 mM MgSO_4 and 145 mM NaCl. *o*-Nitrophenyl β -D-galactopyranoside (ONPG) and *p*-nitrophenyl β -D-galactopyranoside (PNPG) (1 mM) were the substrates used. Before the reaction was started by addition of the enzyme, the mixture was preincubated at 25 °C for at least 10 min. Enzyme activity was determined by the amount of *o*-nitrophenol or *p*-nitrophenol formed per min. Brand new cuvettes or cuvettes soaked in acid were used for the assays to ensure that no active wild-type β -galactosidase remained attached to the

glass of the cuvettes from previous work in the laboratory. Kinetic data of the wild-type and mutated β -galactosidases were obtained by assaying the enzymes at different substrate concentrations. For the Gln- and Val-substituted enzymes, a solution of BSA (2 mg/ml) was added to the reaction system to avoid inactivation of the mutated enzyme during the long periods of incubation (usually 1–6 h) required to detect the weak activity of these enzymes. The substrate analogue, IPTG, and the transition-state analogue inhibitors, 2-deoxy-2-amino-D-galactose and L-ribose, were tested for inhibitory effects. All kinetic parameters were calculated using the relative weighting system discussed by Cornish-Bowden (1976). Methanol is a very good acceptor of β -galactosidase (it can replace water and 'accept' the galactose from the galactosyl form of the enzyme) and it speeds up the reaction of the wild-type enzyme (Shifrin and Hunn, 1969; Viratelle et al., 1969; Deschavanne et al., 1978). The effect of addition of methanol to the assays was measured for each enzyme. Large amounts of methanol (up to 600 mM) were used, but it has been shown previously (Shifrin and Hunn, 1969; Martinez-Bilbao et al., 1991) that such high amounts are not detrimental to the activity of this enzyme.

Stability to heat, c.d. and fluorescence

Each enzyme was incubated (0.5 mg/ml) at 52 °C. Samples were removed periodically, and assayed under normal assay conditions to determine the amount of activity remaining. C.d. and Trp fluorescence of the native proteins were measured at room temperature.

RESULTS

Purity and molecular mass

Figure 1(a) shows that the substituted enzymes were pure (> 98% as estimated by densitometry tracings), and that the mutated enzymes had the same subunit molecular mass as the wild-type β -galactosidase. The enzymes with the substitutions were eluted from a Superose 6 column at the same volume as the wild-type enzyme. Each of the substituted enzymes was isolated on at least three separate occasions. In each case, two separate purifications were carried out starting with two different colonies. The specific activities of the final purified enzymes were essentially the same in each case despite the different starting colonies. The substituted enzymes were also each isolated once using only containers that had first been heated to temperatures above 100 °C and using only new column material, and, again, the specific activities of the purified enzymes were almost the same as when these precautions (designed to ensure that wild-type enzyme would not contaminate the preparations) were not taken.

Kinetic parameters

The kinetic parameters obtained from the assays of the wild-type enzyme and of the three substituted enzymes are exhibited in Table 1. The k_{cat} values of the mutated enzymes for both ONPG and PNPG were much smaller than that of the native enzyme. The K_m values of the substituted enzymes were similar to that of the normal enzyme, the main difference noted being for the enzyme substituted with Asp. The substrate analogue, IPTG, was an effective inhibitor of each mutated enzyme (Table 1). Of the three, it inhibited the E537D mutant the least. The transition-state analogue inhibitor, 2-deoxy-2-aminogalactose (Huber and Gaunt, 1982), inhibited each substituted enzyme slightly more than it inhibited the wild-type enzyme, but the differences were not large. L-ribose, another transition-state analogue inhibitor

Figure 1 (a) Photograph of an SDS/polyacrylamide gel showing the purity of the enzymes and (b) photograph of a native polyacrylamide gel showing that the tetrameric forms of the enzymes migrated similarly

In both gels: W, wild type β -galactosidase; D, E537D β -galactosidase; Q, E537Q β -galactosidase; V, E537V β -galactosidase.

Table 1 Kinetic properties of native and substituted β -galactosidases

NH₂-Gal, 2-deoxy-2-amino-D-galactose.

Constant	Wild-type	E537D	E537Q	E537V
k_{cat} (ONPG) (s ⁻¹)	480	0.63	0.003	0.017
Fold decrease	—	760	160 000	28 000
K_m (ONPG) (mM)	0.12	0.42	0.12	0.12
k_{cat} (PNPG) (s ⁻¹)	120	0.12	0.0004	0.0019
Fold decrease	—	1000	300 000	63 000
K_m (PNPG) (mM)	0.04	0.37	0.05	0.05
K_i (IPTG) (mM)	0.11	0.30	0.12	0.06
K_i (NH ₂ -Gal) (mM)	1.0	0.61	0.41	0.67
K_i (L-ribose) (mM)	0.40	0.50	0.51	0.44

(Huber and Brockbank, 1987), inhibited the three substituted enzymes and the wild-type enzyme by roughly the same amount.

Effect of Mg²⁺ and EDTA

Wild-type β -galactosidase has a requirement for Mg²⁺ or Mn²⁺. As a carboxy group could be a ligand for Mg²⁺ or Mn²⁺, it was thought that the substituted β -galactosidases could be inactive because of a decreased binding affinity for Mg²⁺. Excess Mg²⁺ was therefore added to see if the activity was increased. The addition of a tenfold excess of Mg²⁺ had no effect on the activity of the mutated enzymes. When EDTA (2 mM) was added to the assay mixtures, the activity of each substituted enzyme was reduced to the same extent as that of wild-type β -galactosidase (about an 80 % loss of activity was noted in each case) (results not shown).

Effect of methanol

Figure 2 shows that E537D β -galactosidase was not activated by the addition of methanol. The E537Q and E537V preparations, on the other hand, were activated to about the same extent as the wild-type β -galactosidase.

Stability to heat

Figure 3 shows that the activities of the wild-type, E537Q and

Figure 2 Effect of methanol on the rates of reaction of wild-type and substituted enzymes

The concentration of ONPG was 1 mM. The values are percentages of the activity in the absence of methanol. ○, Wild-type; ×, E537D; △, E537Q; □, E537V.

E537V β -galactosidases were all quite stable at 52 °C. The Asp-substituted enzyme was significantly less stable. Similar effects were noted at 55 °C, but the inactivation was more pronounced.

Conformational properties

Figure 1(b) shows native polyacrylamide gels of the purified proteins. Each of the mutated proteins migrated to the same position as the wild-type β -galactosidase. A small amount of aggregation occurred and this varied somewhat between the enzymes, but aggregation normally varies from preparation to preparation even for wild-type β -galactosidase. C.d. (from 210 to 290 nm) spectra are shown in Figure 4 and fluorescence (excitation at 290 nm and emission between 300 and 400 nm) spectra are shown in Figure 5. The spectra in each case were essentially identical and did not reveal any conformational differences between any of the four enzymes.

Figure 3 Stability to heat of wild-type and substituted β -galactosidases

The enzymes were each incubated at 52 °C, and samples were removed at different times and assayed under normal conditions with 1 mM ONPG. The values are percentages of the activity present before the enzymes were incubated at the elevated temperature. $\circ$, Wild-type; $\times$, E537D; $\triangle$, E537Q; $\square$, E537V.

Figure 4 C.d. spectra of wild-type (.....), E537D (-----), E537Q (——) and E537V (——) β -galactosidases

The spectra were measured with a Jasco J-500A spectropolarimeter at room temperature and at enzyme concentrations of 0.5 mg/ml. The light path length was 1 mm.

2,4-Dinitrophenyl β -D-2-deoxy-2-fluorogalactopyranoside

Dr. S. G. Withers of the Chemistry Department at the University of British Columbia kindly carried out some studies for us with 2,4-dinitrophenyl β -D-2-deoxy-2-fluorogalactopyranoside. The results are preliminary, and more work will be required before hard data can be presented, but when a substoichiometric amount (< 50 % as much reagent as enzyme) of the reagent was added to

Figure 5 Fluorescence spectra of wild-type (.....), E537D (-----), E537Q (——) and E537V (——) β -galactosidases

The spectra were measured with an American Instrument Co. spectrofluorimeter at room temperature and at enzyme concentrations of 0.1 mg/ml. The range was set at 1. The light path length was 1 cm.

E537Q β -galactosidase, the enzyme rapidly lost its activity. Stoichiometric amounts were, however, required for inactivation of E537D β -galactosidase and the inactivation was roughly five times as slow.

DISCUSSION

The Asp-substituted enzyme was made so that the carboxy group would be retained but the effective distance to the substrate would be shorter (assuming that the backbone structure of the enzyme does not change). The substitution by Gln was made to replace the carboxy group with an uncharged side chain very similar in size to Glu. The Val substitution was made to replace Glu with a hydrophobic residue similar in size to Glu. In all three cases, the k_{cat} values of the substituted β -galactosidases were dramatically reduced (Table 1).

The subunit molecular masses of the substituted enzymes, as assessed by mobility on SDS/PAGE, and the native molecular masses, as determined by elution through a size-fractionation column, were essentially identical with the wild-type. The enzymes were therefore of normal size and in the tetrameric form. There were no conformational differences between the enzymes that were significant enough to be detected by native PAGE, c.d. or fluorescence. Adding excess Mg^{2+} did not increase the activity, and adding EDTA reduced the activity of each substituted enzyme and the wild-type enzyme equally, showing that the bivalent metal properties of the enzymes were also similar to those of wild-type. The binding of transition-state analogues was also not significantly altered by the substitutions for Glu-537.

Of the three substituted enzymes, E537D was the most different in terms of its properties. Its k_{cat} values were lowered by a much smaller amount and the K_m value with ONPG as the substrate was about three times that for the wild-type, and, with PNPG as the substrate, the K_m was about ten times as large. The K_m values for the other two substituted enzymes were essentially identical

with those of the wild-type enzyme. In addition, the K_i value for IPTG, a substrate analogue of β -galactosidase, was greater for the Asp-substituted enzyme but was about the same as the wild-type for the other enzymes. Methanol had no effect on the activity of the E537D mutant, whereas it activated the other two enzymes by more or less the same amount as it activated the wild-type enzyme. Methanol is therefore either not a galactosyl acceptor for the E537D mutant or the rate-determining step for that enzyme is such that the rate is not increased by the addition of methanol. Also, of the three substituted enzymes, only the E537D mutant was less stable than the wild-type enzyme.

Every effort was made to protect against contamination by wild-type β -galactosidase. If there was activity present due to wild-type β -galactosidase, it was not because of contamination by a few wild-type *E. coli* organisms among the mutated *E. coli* that were the source of the enzymes. Two different colonies were chosen to start the cultures used for two separate preparations of each substituted enzyme, and the k_{cat} values of the enzymes from these two different preparations were the same in each case. It is unlikely that two different colonies would both be contaminated by the same amount of wild-type *E. coli*. The activity also did not arise from contamination by wild-type β -galactosidase of glassware and other material used during the purifications. For each enzyme, one purification was carried out in which extreme care was taken to use only autoclaved glassware (to destroy any wild-type β -galactosidase molecules that might have been attached to the glass from previous work in the laboratory) and to use new column material, and yet the k_{cat} values were the same as when less care was taken.

It is, however, very probable (despite the precautions described above) that most of the small amount of activity found for the Gln- and Val-substituted enzymes was not from the substituted enzymes themselves but from a few molecules of wild-type enzyme present in the substituted enzyme preparations. These were probably produced by reading errors (Edelmann and Gallant, 1977). Especially significant in this regard are the findings that the K_m values, the K_i values for IPTG, the effect of methanol and the stability to heat for these two enzymes were very similar to those of the wild-type enzyme. The results of the study carried out for us by Dr. S. G. Withers, although preliminary, also suggest this. The activity of the E537Q mutant preparation was rapidly lost when only substoichiometric amounts of the reagent 2,4-dinitrophenyl β -D-2-deoxy-2-fluoro-

galactopyranoside were used. For the E537D mutant the activity loss was much slower and stoichiometric amounts of the reagent were needed for inactivation because the reagent was probably reacting with the carboxy group of the Asp. If the activities of the E537Q and E537V mutant preparations are indeed mainly a result of a few wild-type molecules mixed with large amounts of inactive substituted enzymes, the effect of substituting for Glu-537 is even more significant. Essentially it seems that the E537Q and E537V mutants of β -galactosidase are probably totally inactive.

Overall, the data show that substitutions for Glu-537 dramatically decrease the catalytic activity of β -galactosidase. Thus Glu-537 is probably a very important part of the catalytic machinery of the enzyme.

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